## Dimerization and signaling of the chemokine receptors CXCR3 and CCX-CKR

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# Chapter 7

General discussion

GPCR homo- and heteromerization modulate receptor ontogeny, ligand binding characteristics, signaling pathway specificity and endocytic trafficking.[1] Chapter 2 of this thesis provides an overview of these functional consequences of dimerization and the challenges in discerning them from other, indirect forms of crosstalk between GPCRs. Dimerization also occurs within the large family of chemokine receptors and may have consequences for chemotaxis towards chemokine gradients as wells as drugs targeting these receptors.[2] Most chemokine receptors are able to bind multiple chemokines and the different chemokines may bias the receptor to activate different signaling pathways. Additionally, glycosaminoglycans and atypical chemokine receptors regulate chemokine concentration and localization.[3] The chemokine receptor CXCR3 and its ligands CXCL9, CXCL10 and CXCL11 are potential drug targets in auto-immune disease and organ transplant rejection.[4,5] Despite considerable interest in CXCR3 and its chemokines, few reports have addressed CXCR3 dimerization in detail.[6,7] An obvious potential dimerization partner for CXCR3 is the widely-expressed chemokine receptor CXCR4, which has been shown to dimerize with chemokine receptors as well as other GPCR subtypes.[2,8-14] CXCR3 and CXCR4 are both expressed on lymphocytes, endothelial cells and cancer cells and the cognate chemokines of CXCR3 and CXCR4 have been shown to potentiate each other's activity *in vivo*.[15-19] This thesis describes our investigations into the regulation of chemokine receptor CXCR3 function through dimerization and crosstalk with CXCR4 and the atypical chemokine receptor CCX-CKR.

**Dimerization and negative ligand binding co-operativity.** The potential of GPCR dimerization to affect receptor function has been firmly established. Nevertheless, interactions between GPCRs are selective and not all receptor subtypes are able to interact with each other.[20] In response to a spate of publications demonstrating GPCR dimerization in heterologous expression systems, the importance of identifying GPCR dimers at physiological receptor expression levels, *e.g.* in native tissues or titrated heterologous expression, has been

highlighted by Pin *et al*. (2007).[21] Additionally, the importance of functional and physiological relevance of GPCR dimers has also been emphasized.[22] Functional effects of GPCR dimerization have been described in numerous publications. However, in many cases it is difficult to discern whether the responses observed on co-expression of GPCRs are the result of direct physical interactions between receptors or of a different, indirect mechanism of receptor crosstalk.[23]

Effects of CXCR4, CCR2b and CCR5 heterodimerization and hetero-oligomerization on ligand binding has been demonstrated in heterologous expression systems as well as human primary lymphocytes and monocytes.[2,9,24] Negative chemokine binding co-operativity was observed for heteromers of these three chemokine receptors. Negative ligand binding co-operativity at the CXCR4-CCR5-CCR2b heteromers is not limited to the endogenous chemokine agonists, but extends to synthetic antagonists of the two protomers, thereby trans-inhibiting chemotaxis mediated by the other protomer. Furthermore, the synthetic antagonist of one protomer trans-inhibited chemotaxis induced by the chemokine of the second protomer.[2,9] Functional consequences downstream of ligand binding have also been reported, showing that cell surface trafficking and signaling differ between homo- and heterodimers of CXCR4 and CCR5 or CXCR7.[10,25-27]

In chapter 3, we show that CXCR3 and CXCR4 form homo- and heterodimers at the cell surface in transiently transfected HEK293T cells with approximately equal propensity. Negative binding co-operativity was observed between chemokine and small molecule agonists of CXCR3 and CXCR4 in membrane preparations expressing both receptors. In contrast to previously described chemokine receptor heterodimers,[2,8] synthetic antagonists of CXCR3 (TAK-779) and CXCR4 (AMD3100) did not trans-inhibit binding of radiolabeled CXCL12 or CXCL10, respectively. Membrane preparations are a convenient and commonly used source of GPCRs for radioligand binding experiments. For some GPCR-agonist interactions, G protein uncoupling, *e.g.* by an excess of guanine nucleotides, decreases the affinity of the agonist for the receptor to such a degree that meaningful ligand binding cannot be measured. Therefore, consideration must be given to the possibility that trans-

inhibition of binding to membrane preparations between agonists of GPCRs that share the same pool of G protein subtypes may also result from G protein scavenging, which is artificially enhanced by the lack of free GTP under these experimental conditions.[28] An activated agonist-bound GPCR couples to the G protein and induces GDP dissociation from the G protein. In the absence of free GTP, such as in membrane preparations, the agonist, receptor and nucleotide-free G protein form a pseudo-irreversible complex.[29] If agonist-bound GPCRs that couple to the same G protein subtype outnumber G proteins, G protein scavenging will occur, mimicking negative agonist binding co-operativity under equilibrium binding conditions. To exclude the possibility that negative agonist binding co-operativity observed in membranes is actually an artefact caused by G protein scavenging, equilibrium radioligand binding studies in membrane preparations should be complemented with or replaced by control experiments. Evidence to rule out or reinforce may be obtained from equilibrium binding studies in membrane preparations in the presence of an excess of guanine nucleotides (GDP, GTP or nonhydrolyzable GTP analogue) to uncouple G proteins from GPCRs; equilibrium binding studies in whole cells; or radioligand dissociation experiments. High affinity binding of CXCL10 and CXCL12 to CXCR3 and CXCR4, respectively, is dependent on $G_i$ proteins.[14,30] Therefore, in Chapter 3, we measured radioligand dissociation from membrane preparations and radioligand equilibrium binding to intact cells to confirm the observed negative ligand binding co-operativity between CXCR3 and CXCR4 agonists. However, the results from these two control experiments contradicted each other. On co-expression of CXCR3 and CXCR4, the CXCL12 dissociation rate is accelerated by CXCL10 agonists, supporting the hypothesis that negative agonist binding co-operativity exists in CXCR3/CXCR4 heteromers. As in the CXCL12 dissociation experimental setup radiolabeled CXCL12 is pre-loaded onto the membranes prior to addition of the cold ligand, it is assumed that any effect of the cold ligand cannot result from G protein scavenging and, therefore, must be due to an allosteric interaction between binding sites of the cold and radiolabeled ligands. However, although the nucleotide-free G protein binds with

high affinity to the agonist-bound receptor, this interaction is still subject to a thermodynamic equilibrium and G protein switching from one GPCR to another may be facilitated by close proximity of the receptors, such as for two protomers of a dimer. Springael *et al*. (2006) have proposed an explanation of similar results for CCR2b-CCR5 heterodimers.[8] The increased dissociation rate of the pre-bound chemokine may be due to G protein scavenging within the heterodimer. An excess of guanine nucleotides produces a response similar to that of an excess of cold chemokine in a radioligand dissociation experiment. These results were proposed to indicate a role for G protein scavenging in enhancing the radioligand dissociation rate. However, when agonist binding and G protein coupling are interdependent, as is the case for CXCR3 and CXCR4, it is very difficult to discern between negative ligand binding co-operativity and G protein scavenging. In the control experiment of equilibrium radioligand binding in whole cells, negative binding co-operativity was not observed, even though CXCR3-CXCR4 heteromers are expressed on the cell surface and specific binding of both CXCL10 and CXCL12 was readily observed. At present, we cannot reconcile the results from the two different types of control experiments performed to confirm negative chemokine binding co-operativity within CXCR3-CXCR4 heterodimers. Although performed at low temperatures, equilibrium binding experiments performed with whole cells arguably reflect the physiological environment for receptor binding more closely than binding experiments with membrane preparations. Therefore, the lack of negative ligand binding co-operativity in whole cells may indicate that negative ligand binding co-operativity is not physiologically relevant for CXCR3-CXCR4 heterodimers. However, it remains to be explained why the outcome of radioligand dissociation experiments do not support these findings.

In contrast to radioligand binding to CXCR3 and CXCR4, specific CCL19 binding cannot be detected in membranes prepared from cells expressing CCX-CKR (data not shown). However, specific CCL19 binding is readily detected in whole cells. It is not clear why CCX-CKR chemokine binding is lost during the membrane preparation process. CCX-CKR may require cytosolic proteins for high affinity ligand

binding, which are removed by the membrane preparation process. In analogy to the dependence on G proteins of specific GPCR-agonist pairs for high affinity binding, β-arrestins are also able to enhance ligand binding.[31] In contrast to CXCR3-CXCR4 heteromers (Chapter 3), negative ligand binding co-operativity was detected between chemokines binding to CXCR3-CCX-CKR heteromers on intact cells (Chapter 4). As Nijmeijer *et al*. (2010) have shown, G protein scavenging is not limited to membrane preparations, but may also occur in whole cells with physiologically relevant consequences.[14] The viral chemokine receptor BILF1 exhibits strong constitutive activation of $G_i$ proteins. CXCL12 binding to CXCR4 is inhibited by BILF1 in a $G_i$-dependent manner.[14] However, whereas CXCL10 binding to CXCR3 is dependent on $G_i$ proteins, chemokine affinity for CCX-CKR is not affected by the $G_i$ inhibitor PTX. As CXCR3 is not known to activate G proteins other than $G\alpha_i$ family G proteins, this suggests that ligand binding is affected by an allosteric interaction within the CXCR3-CCX-CKR heterodimer. Given that similar chemokine affinities for CXCR3 are detected both in membrane preparations and whole cells, CXCR3 agonist binding appears to be independent of β-arrestins. In Chapter 5, we show that CCX-CKR interacts with $G_i$ proteins, though it is not yet clear whether the CCX-CKR-$G_i$ protein interaction is constitutive, ligand-induced or a combination of these two. It is conceivable that CCX-CKR coupling to $G_i$ limits the availability of this G protein for CXCR3, and at the same time, β-arrestin recruitment to CXCR3 limits β-arrestin availability for CCX-CKR. It would be interesting to investigate whether CCX-CKR chemokine binding is sensitive to β-arrestins to support this possibility. However, the binding experiments were performed at low temperatures to prevent the enzymatic activity required for receptor internalization (Chapter 4). Efficient β-arrestin recruitment generally requires GPCR phosphorylation, which is also an enzymatic process and unlikely to occur efficiently at these low temperatures. Thus, it remains to be determined whether β-arrestin plays a role in trans-inhibition of ligand binding between CXCR3 and CCX-CKR.

Crosstalk between GPCRs at the level of negative ligand binding co-operativity and at more downstream levels of the signal transduction pathways may be interdependent on each other. It is clear that determining the role of allosteric intradimer interactions between GPCRs in trans-inhibition of ligand binding requires information in addition to radioligand binding data. Vilardaga *et al.*, (2008) have demonstrated an intradimer allosteric interaction between the orthosteric binding sites of the $\alpha_{2A}$ adrenergic receptor and the μ opioid receptor using intraprotomer FRET.[32] Although this approach requires GPCRs fused to fluorescent proteins and cannot be used to study dimers in native tissues, it is the most unequivocal evidence of intradimer allosteric interactions available to date.

**Functional effects of dimerization versus downstream crosstalk.** Functional effects of apparent negative binding co-operativity between CXCR3 and CXCR4 chemokines could not be detected in chemotaxis experiments with transiently transfected murine B lymphoblast L1.2 cells (data not shown). These results are similar to results from CXCR4-CCR2 dimerization studies. CXCR4 and CCR2-induced chemotaxis and calcium mobilization could be trans-inhibited by antagonists, but not chemokines, even though negative binding co-operativity was observed between chemokines of CXCR4 and CCR2. It is difficult to define functional consequences of negative binding co-operativity between GPCRs that activate the same G protein subtypes, as is the case for $G_i$ activation by chemokine receptor family members. Moreover, chemotaxis is a particularly complex functional readout to interpret due to the bell-shaped concentration-response curves. Therefore, the effect of an antagonist of one chemokine receptor on the function of a chemokine at a second receptor is easier to distinguish than the effect of a chemokine.

In Chapter 3, we show that β-arrestins are cross-recruited to CXCR3 in response to treatment with CXCR4 chemokine and *vice versa*. Although strengthening the evidence of the existence of CXCR3-CXCR4 heterodimers, it is not clear whether β-arrestin recruitment to CXCR3-CXCR4 heterodimers has physiological consequences

that differ from responses to β-arrestin recruitment to CXCR3 and CXCR4 monomers or homodimers. Interestingly, co-stimulation with CXCL11 and CXCL12 potentiated the signal in the GPCR-HIT assay in comparison to stimulation with the individual chemokines. This does not appear to support negative binding co-operativity between CXCL11 and CXCL12 at the CXCR3-CXCR4 heteromer. However, potentiation of the BRET signal may reflect enhanced β-arrestin2 recruitment to the CXCR3-CXCR4 heteromer, but may also be caused by changes in the distance between and relative orientation of the BRET donor and acceptor. CXCR4 is known to activate signaling pathways downstream of β-arrestin and requires β-arrestin to mediate efficient chemotaxis.[33] Although CXCR3 is known to recruit β-arrestins in response chemokines, CXCR3-mediated signaling through β-arrestin has not been described in literature.[34,35] It would be interesting to investigate whether CXCR3-CXCR4 heterodimerization affects β-arrestin-dependent signaling through CXCR4. It should be investigated whether CXCR3 and CXCR4 are subject to cross-phosphorylation by GRKs, cross-desensitization and cross-internalization. Moreover, the role of CXCR3-CXCR4 heteromerization in the potentiation of immune cell migration *in vivo* and *in vitro* seen on co-stimulation with CXCL11 and CXCL12 would be very interesting to study.[18,19]

Interestingly, co-expression of CXCR3 with CCX-CKR decreased specific binding of both CXCL10 and CCL19 in comparison to cells expressing only CXCR3 or CCX-CKR, respectively. In line with the decreased CCL19 binding, cell surface expression of CCX-CKR was decreased in the presence of CXCR3 (Fig. 1). In contrast, decreased CXCL10 binding was not matched by decreased CXCR3 cell surface expression, as CXCR3 is localized predominantly at the cell surface at similar expression levels in both the absence and presence of CCX-CKR (Fig. 1). Indeed, decreased numbers of CXCR3 would result in loss of equal fractions binding sites for both CXCL10 and CXCL11. However, CXCL11 binding in cells expressing CXCR3 was not affected by the presence of CCX-CKR to the same degree as CXCL10 binding. Paradoxically, the affinity of CXCL10 for CXCR3 was not affected by the presence of CCX-CKR.

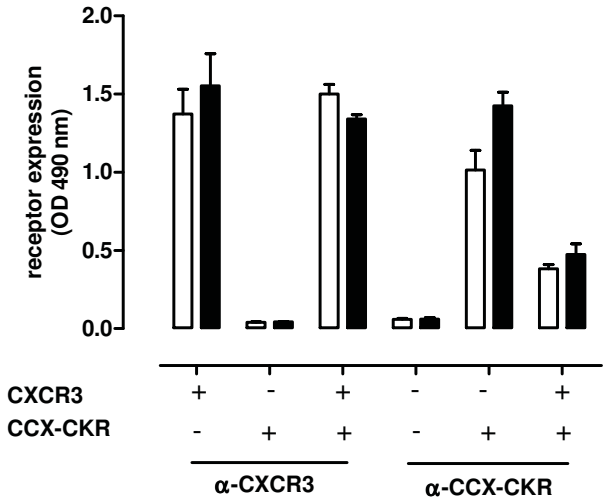


**Figure 1. Co-expression of CXCR3 and CCX-CKR decreases CCX-CKR expression but does not affect CXCR3 expression.** HEK293T cells were transiently transfected with CXCR3, CCX-CKR or co-transfected with both receptors as indicated in the figure. Cell surface (empty bars) and total (filled bars) receptor expression was determined using ELISA in intact and permeabilized cells, respectively. Elisa was performed with monoclonal antibodies targeting CXCR3 (clone 49801; R&D Systems; Minneapolis, MN) and CCX-CKR (clone 2F11; a kind gift from Dr. Chiba, Tokyo University of Science, Japan[48]) as described in Chapter 5.

The combined data suggest that CCX-CKR increases the fraction of CXCR3 receptors with a conformation for which CXCL10 has an affinity that is too low for detection of CXCL10 binding. This would explain the specific loss of CXCL10 binding sites without affecting the affinity measured for CXCL10 or CXCL11 binding.

It is not clear whether CXCR3-CCX-CKR heterodimerization underlies the inhibitory effect of CCX-CKR on CXCL10-induced chemotaxis, or whether this inhibitory effect occurs further downstream in the CXCR3 signaling cascade. Chemotaxis towards CXCL11 should be investigated to exclude the possibility that the effect of CCX-CKR on CXCR3 function is limited to CXCL10-induced responses. The observations that

CCR5 does not dimerize with CCX-CKR and that CCR5-mediated chemotaxis is not affected by the presence of CCX-CKR indicate that CCX-CKR does not have an inhibitory effect on chemotaxis in general. To support the apparent correlation between dimerization and inhibition of chemotaxis, dimerization of CCX-CKR with the panel of chemokine receptors for the ligands tested in the chemotaxis assay should be determined. Interestingly, CCX-CKR was found to interact with $G_i$ proteins (Chapter 5). It remains to be determined whether the interaction of CCX-CKR with $G_i$ proteins is ligand-induced. A constitutive interaction between CCX-CKR and $G_i$ could explain the effect of CCX-CKR on CXCL10 binding and chemotaxis and its lack of effect on CCR5-mediated chemotaxis. Dimerization of CXCR3 and Gi-bound CCX-CKR may prevent agonist-induced $G_i$ coupling to CXCR3 through steric hindrance. As the CXCR4-BILF1 and CXCR3-CCX-CKR heterodimers demonstrate, GPCR dimerization and G protein coupling to GPCRs can be intertwined and have inextricable effects on GPCR signaling.

CCX-CKR is the third atypical chemokine known to heterodimerize with GPCRs. The Duffy antigen receptor for chemokines (DARC) dimerizes with CCR5. Heterodimerization with DARC inhibits CCR5-mediated chemotaxis and $Ca^{2+}$ mobilization.[36] Interestingly, DARC does not affect chemokine-induced CCR5 internalization and, therefore, apparently does not affect affinity of chemokines for CCR5. Co-internalization of CCR5 and DARC was not investigated and it would be interesting to find out whether CCR5 and DARC, as well as CXCR3 and CCX-CKR, are co-internalized. CXCR7 forms heterodimers with CXCR4, impairing CXCR4 $G_i$ activation and biasing CXCR4 to activate β-arrestin-dependent signaling pathways.[25,37] Taken together, the current literature and our findings presented in Chapter 4 suggest that atypical chemokine receptor heterodimerization may be an important mechanism for regulation of chemokine receptor activity besides their role in the regulation of chemokine availability.

**CCX-CKR signaling.** In light of the inhibitory effect of CCX-CKR on chemotaxis towards various chemokines (Chapter 4), we investigated the potential of CCX-CKR

to activate intracellular signaling pathways. No CCL19, CCL21 or CCL25-induced signaling was detected for CCX-CKR using label-free impedance measurements (Fig. 2), even though CXCR3-mediated signaling could be detected in the label-free assay (Chapter 6).

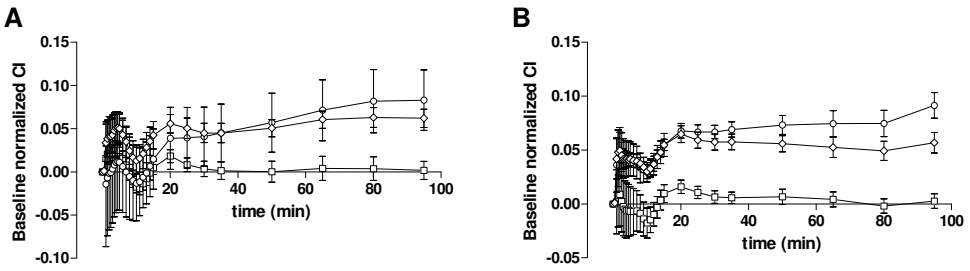


**Figure 2. CCL19, CCL21 and CCL25 do not signal through CCX-CKR.** Cell Index (CI) traces of **(A)** mock transfected HEK293 cells and **(B)** HEK293 cells stably expressing CCX-CKR. At time = 0 min, 100 nM CCL19, CCL21 or CCL25 was added to the cells. Data shown are averages ± SEM from a single experiment peformed in triplicate. Data were corrected for baseline values (vehicle-treated cells) and normalized to the CI at time = 0 min.

However, subsequent investigations using reporter gene assays showed that CCL19 induces CCX-CKR-mediated activation of the transcription factor cAMP responsive element-binding protein (CREB) (Chapter 5). CCX-CKR-mediated CREB activation could only be detected in the presence of PTX and this signal was potentiated by the adenylate cyclase activator forskolin. CREB is a transcription factor linked to a broad range of cellular processes, including cell proliferation and differentiation.[38] Various stimuli induce phosphorylation of CREB, which may the bind to CRE sequences in the promoters of target genes, enhancing their transcription. CREB activation generally involves $G\alpha_s$, activation of which results in phosphorylation of CREB by the cAMP-dependent protein kinase A (PKA). It is now known that CREB can also be phosphorylated by a host of serine/threonine kinases, such as PKC and calmodulin-dependent kinases, the activation of which

depends on an increase in intracellular $Ca^{2+}$ concentration.[38,39] Although CREB can be activated through signal transduction pathways that do not involve cAMP, the enhancement of forskolin-induced CREB activity indicates that the CCX-CKR-mediated signal is transduced through $G\alpha_s$.[40,41] The physiological relevance of CCX-CKR signaling through $G_s$ is far from clear, given that - even in a highly sensitive reporter gene assay - the $G_s$ signal is detected only in the presence of two exogenous compounds (PTX and forskolin). The interaction of CCX-CKR with $G_i$ proteins occurs may compete with $G_i$-activating GPCRs. In this manner, CCX-CKR may be involved in the regulation of GPCRs other than those with which it shares its ligands. To determine whether the CCX-CKR interaction with $G_i$ is constitutive or ligand-induced, and whether CCX-CKR indeed activates $G_s$ in response to chemokine stimulation, co-immunoprecipitation and BRET studies could be performed. Additionally, reassessment of CCX-CKR-mediated signaling using impedance measurements should be performed in the presence of PTX. These experiments will provide additional evidence on the role of $G_s$ in CCX-CKR signaling, as $G_s$ activation results in a characteristic decrease in impedance.[42]

In addition to interactions with G proteins, we have shown that CCX-CKR recruits β-arrestins in a CCL chemokine-dependent manner (Chapter 5). Comerford *et al*. (2006) have previously demonstrated that internalization of CCX-CKR is dependent on caveolin-1 and does not require β-arrestins and clathrin-coated pits (CCPs).[43] As we have used a similar cell lines for our experiments (HEK293T *versus* HEK293 used by Comerford *et al*.), it is unlikely that the ability to recruit β-arrestin or the use of internalization pathways differ between these cell lines. Indeed, our combined data serve as a reminder to exercise caution in equating β-arrestin recruitment to β-arrestin/CCP-dependent internalization. Similarly to CCX-CKR, the atypical chemokine receptor D6 recruits β-arrestins and was initially thought to internalize through CCPs.[44] Further studies have revealed that although D6 constitutively interacts with β-arrestin, rather than promote internalization, β-arrestin has an inhibitory action on chemokine scavenging by D6.[45] However, it must be taken into consideration that the internalization route utilized by a GPCR

may differ depending on the cellular background.[46] Moreover, GPCR internalization may a combination of different pathways in the same cell.[46] Additionally, an association of CCX-CKR with caveolae, which is suggested by the dependence of CCX-CKR internalization on caveolin-1, provides an interesting potential mechanism that may facilitate $G_s$ activation by CCX-CKR.[43,47]

In conclusion, we have studied CXCR3 heteromerization and the role of crosstalk in the regulation of chemokine function. It remains challenging to define whether crosstalk between GPCRs occurs through direct physical interactions or indirect interactions, especially as the focus of research shifts towards receptors in their native environment. We have demonstrated that CXCR3 heteromerizes with CXCR4 and the atypical chemokine receptor CCX-CKR. Although we were not able to conclusively demonstrate an effect of CXCR3-CXCR4 heteromerization on ligand binding, a number of potential physiological consequences remain to be explored for this heteromer. Our studies of CXCR3-CCX-CKR heteromerization and CCX-CKR signaling have brought new insights into the regulation of chemokine receptor signaling through CCX-CKR and has opened up new avenues of research to be explored for atypical chemokine receptors.

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